May 7, 1974 E. E. SCHMITT 3,809,617

DEVICE FOR DETECTING ANTICHOLINESTERASE MATERIALS

Filed Nov. 15, 1972

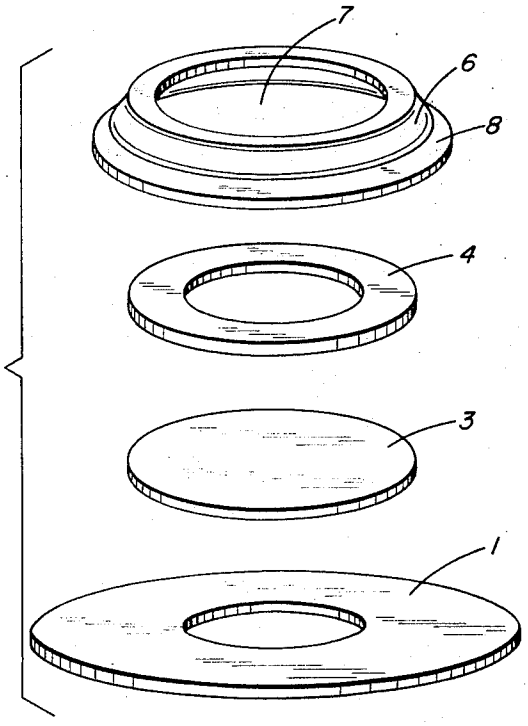


FIG. 3

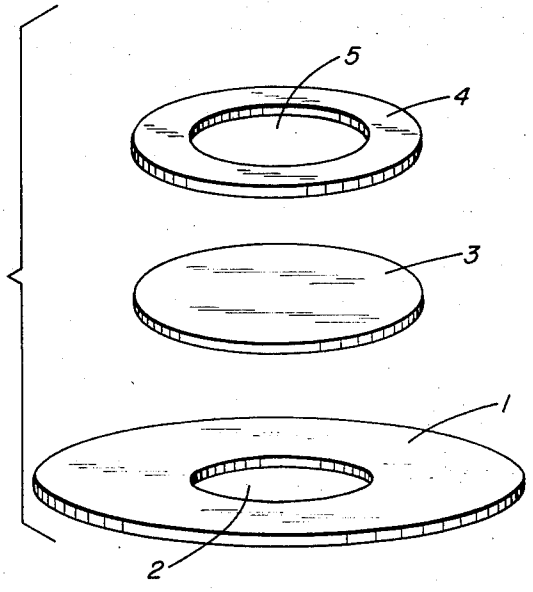


FIG. 1

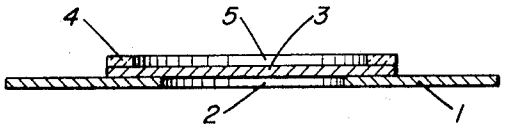


FIG. 2

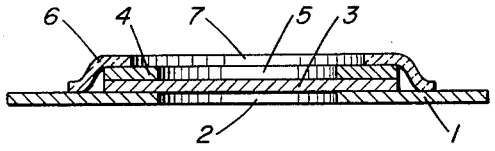


FIG. 4

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DEVICE FOR DETECTING ANTICHOLINESTERASE MATERIALS

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Int. Cl. C12k 1/10
U.S. Cl. 195—127 10 Claims

ABSTRACT OF THE DISCLOSURE

An article of manufacture comprising, in superimposed relationship, (A) a self-supporting layer, (B) a cellulosic element having sulfited polyacrolein bound cholinesterase dispersed throughout the interstices thereof and (C) an active cholinesterase free cellulosic layer having the same physical properties of color and texture as the cellulosic element (B) after both have been wetted with buffer and an appropriate detection solution.

Background of the invention

There has existed for a considerable period of time means for conducting tests designed to detect the presence of anticholinesterase materials in liquids and gases. The most widely used device for detecting these materials consists of a fibrous mat, usually produced from glass fibers, which has horse serum cholinesterase impregnated therein. These devices are generally used in the field in the detection of anticholinesterase materials e.g. nerve gas, which are fatal if inhaled or otherwise contacted by human beings or animals and they are almost always in the form of kits which contain, in addition to the mat, a series of packaged liquids which must be used to wet the mat before detection of the anticholinesterase materials is possible. In normal use, the cholinesterase impregnated glass mat is wetted with a standard "buffer" solution at a pH of about 7.0 to 7.5 and the thus wetted mat is exposed to the environment to be tested. It is then contacted with 2,6-dichloroindophenyl acetate. The wetted spot is colorless until the cholinesterase in the mat hydrolyzes the acetate thereby resulting in the formation of a blue color on the wetted mat. The blue color affirms the retention of enzyme activity and hence the absence of anticholinesterase material. When contact between the device and a anticholinesterase material is effected, the impregnated cholinesterase will deactivate and the wet spot will therefore remain colorless.

One of two very serious deficiencies of the prior art devices is that fact that, since the cholinesterase is not immobilized but merely impregnated into the glass fiber mat, when the mat is wetted, the cholinesterase tends to chromatograph toward the edges of the wet spot. This migration of the cholinesterase thus results in a non-uniform color development over the area of the wetted surface even when no anticholinesterase materials are encountered since the center thereof contains little or no cholinesterase and therefore remains colorless while the outer edges turn blue. It is then very difficult, especially for unskilled personnel, to determine whether anticholinesterase materials are present or not.

A further deficiency of the existing detection devices is the fact that the operator has no standardized color to which to refer after the various liquids of the kit have been dispensed onto the glass fiber mat. Therefore, it is very difficult to ascertain whether a color change has actually taken place, especially when the quantity of the anticholinesterase materials in the area under suspicion is very low.

Summary

I have now discovered a new and novel detection means whereby both of the above-enumerated deficiencies have been overcome. Utilizing my novel detection means, one can ascertain, with a high degree of accuracy, whether any anticholinesterase materials exist in any given suspected area or not.

Uniform wetting by both the "buffer" solution and the substrate is accomplished with an accompanying uniform color change across the entire wetted area at even very low concentrations of cholinesterase. Furthermore, when the concentration of anticholinesterase material is also very low, my novel device enables the operator to compare the newly formed color with the original color of the wetted area. The device thereby enables a clear, positive test which is easily read and interpreted even by relatively inexperienced personnel.

Description of the drawing

FIG. 1 depicts an exploded view of my novel device wherein an outer layer is positioned above a circular disc and a bottom support layer.

FIG. 2 depicts the novel device of the instant invention wherein the components of FIG. 1 are assembled in superimposed relationship.

FIG. 3 also depicts an exploded view of my preferred novel device wherein two outer layers are positioned above the circular disc and bottom support layer.

FIG. 4 depicts the components of FIG. 3 in assembled, superimposed relationship.

Description of the invention including preferred embodiment

As mentioned briefly above, my novel articles of manufacture in their simplest form comprise, in superimposed relationship, (A) a holder or supporting means, (B) a cellulosic element having cholinesterase bound to sulfited polyacrolein dispersed throughout the interstices thereof and (C) an active cholinesterase free cellulosic layer having a central hole therein and the same physical properties of color and texture as the cellulosic element (B) after both (B) and (C) have been wetted. In a preferred embodiment, a second layer (D) is superimposed upon said first layer (C).

With reference to said drawing, FIG. 1 depicts the broadest configuration of my novel devices wheerin **1** represents a holder or self-supporting means, preferably having a perforation **2** therein. Immediately above said holder is an element **3** which is composed of a cellulosic sheet having active cholinesterase bound to sulfited polyacrolein dispersed throughout the interstices thereof. Layer **4** is produced from the same material as element **3** except that it contains no active cholinesterase and possesses a central perforation **5** preferably of the same diameter as **2**. In FIG. 3, top layer **6** is preferably of inverted dish design and serves to retain element **3** and layer **4** in position on holder **1**. It too is perforated as shown at **7**.

Holder or supporting means **1** can be prepared from any self-supporting material such as cardboard, wood, metal, glass, ceramic and the like and serves as a support for element **3** and layers **4** and **6**. The support is preferably perforated as shown at **2** so that element **3** is observable through both sides of the complete device. Element **3** and layer **4** can be merely placed over the perforation of substrate **1** and can then be attached to each other and holder **1** around the peripheries thereof to hold element **3** and layer **4** in place and maintain the integrity of the device by using an adhesive, fusing, clipping, or otherwise clamping them together. If layer **6** is used, it alone maye be attached to means **1** at its periphery, as described above.

Element **3**, as mentioned above, is formed from a cellulosic sheet which has active cholinesterase bound to hydrophilic, sulfited polyacrolein dispersed throughout the

interstices thereof. The sulfited polyacrolein having horse serum cholinesterase bound thereto forms no part of the instant invention per se and can be produced by sulfiting and cross-linking polyacrolein to render it hydrophilic yet insoluble, as discussed at length in Ser. No. 306,780 filed concurrently herewith by Edward Emil Schmitt and Richard Carl Capozza, entitled Method of Detecting Anti-cholinesterase Materials, and hereby incorporated herein by reference.

By "hydrophilic" is meant that the resultant polymer is swellable in or capable of taking up water. The sulfited polyacrolein is water-insoluble by virtue of its cross-linked nature but may take up and retain water such as by absorption, adsorption etc.

In general, the hydrophilic, sulfited polyacrolein is accomplished as follows. Sulfiting is conducted by treating the polyacrolein with a suitable material which imparts a sulfite group onto the polymer chain such as a sulfite per se, a hydrosulfite, a bisulfite, sulfurous acid and the like. Specifically, alkali metal or alkaline earth metal compounds and salts such as sodium, potassium, calcium, ammonium etc. sulfites, bisulfites, hydrosulfites etc. can be used. The reaction is generally run at 25–90° C. at atmospheric pressure, a more precise method being disclosed in U.S. Pat. Nos. 2,657,192 and 3,271,334, also hereby incorporated herein by reference. By the term "sulfited" is meant that the polyacrolein has been contacted with a sulfite so as to modify it and should not be construed to necessarily mean that any added sulfite groups per se remain on the polymer after cholinesterase binding, although such may be the case.

After the sulfite treatment, the sulfited polyacrolein is insolubilized such as by chemical cross-linking with a cross-linking agent or an immobilization agent useful for this purpose. For example, the procedure of U.S. Pat. No. 3,459,710, incorporataed herein by reference, can be followed. Using this method, the sulfited polyacrolein is contacted with, for example, a diamine such as ethylene diamine, tetramethylene diamine, 1,6 - hexamethylene diamine, N-methylethylene diamine etc. at 0–150° C. in a solvent. Grafting of the sulfited polyacrolein can also be used to insolubilize the polymer in addition to reacting it with such agents as 4-aminophenyl sulfide hydrochloride salt.

The cholinesterase is bound to the hydrophilic, sulfited polyacrolein at a temperature below that at which it, the enzyme, is deactivated. Generally, temperatures below 75° C., preferably 5–65° C. should be used. The binding is carried out in the presence of buffers (pH—7.0–7.5) and with mild agitation. The binding is usually accomplished in the presence of water since organic solvents tend to inactivate the cholinesterase.

The bound cholinesterase material is dispersed throughout the interstices of the cellulosic sheet in amounts ranging from about 1.0% to about 60.0%, by weight, based on the weight of the sheet, the sulfited polyacrolein having from about 0.1 to about 100, preferably 0.2–50.0, units of activity per ½ inch disc at a normal paper sheet thickness of from about 0.5 mils to about 25 mils.

Any cellulosic paper material may be used to form the cellulosic elements of the novel devices of the present invention. The cellulosic paper may be made from all types of fiber stocks, including those of poor quality, such as oak, poplar and yellow birch and those of extremely short fiber length, as well as those of long fiber length and of good quality derivation, such as spruce and hemlock. A wide variety of fibrous cellulosic material used in the preparation of paper, board, and the like may be used such as kraft pulp, rag pulp, ground wood, sulfite pulp, alpha pulp and the like. Similarly, other forms of paper-forming fibrous cellulose such as cotton linters, linen, and the like may be employed. These materials may be used alone or in admixture with fibers from other sources such as jute, hemp, sisal, strings, chopped canvas and other material either cellulosic or non-cellulosic.

It is further stressed that the cellulosic element 3 may also be obtained from bleached or unbleached kraft, bleached or unbleached sulfited or bleached or unbleached semi-chemical pulps. In addition, the element may be made from mixtures of cellulosic paper-forming pulps with up to 10% of such other fibers as glass fibers, synthetic fibers and the like.

For most purposes it is preferred that the starting cellulosic paper be unsized and generally free of added resins. However, for some purposes, it may be desirable to employ as the paper base sheet, a porous, high wet strength paper such as may be obtained by incorporating into the paper from about 0.5 to 5.0%, by weight, based on the weight of the fibers, of a thermosetting aminoplast resin as a urea-formaldehyde resin, a melamine-formaldehyde resin and the like. Such wet strength cellulosic papers are obtained in the conventional way by the use of such a resin applied to the pulp suspension.

The method of making the cellulose element 3 used in my novel devices is not critical and any known paper-making process may be employed. In the normal manufacture of paper, for example, cellulosic fibers such as those derived from wood pulp are beaten in water to disperse the fibers therein and to reduce them to a length and fineness suitable for paper making. During the beating operation the cellulosic fibers fibrillate to produce minute tendrils which serve to interlock the fibers together when they are deposited on the forming screen of the paper making machine to make a sheet therefrom.

The sulfited polyacrolein bound cholinesterase may be added to the cellulosic paper anytime during the production thereof. Therefore, it is within the scope of the present invention to prepare a beater pulp of paper-making cellulosic fibers of any convenient consistency. To this can be added the sulfited polyacrolein bound cholinesterase. The suspension is then agitated gently to distribute the material uniformly therethrough and the aqueous suspension is then sheeted, preferably at a pH of between 4.5 and 6.0, to form a wet, water-laid web containing the bound cholinesterase. The web is then dried, preferably in air or under vacuum. Vacuum drying with a desiccant of calcium chloride for 6 days has proven effective. Drying at elevated temperatures is to be avoided since high temperatures tend to deactivate the cholinesterase and render the disposed material useless. In general, it is preferred that less than about 0.5% of residual water be retained in the final sheet.

In paper-making mills where various pigments are added at the beater, the bound cholinesterase may be added therewith or at any point more than about one minute from the wire. In mills where the pulp suspension is given heavy refining, the bound cholinesterase may be added to the beater, tot he refiner effluent or to the screen effluent sufficiently ahead of the wire so that deposition becomes substantially complete before sheeting. Thus the application of the bound cholinesterase may be easily adapted to most types of paper or mill conditions.

The material from which layer 4 is produced differs from that used to manufacture element 3 only in the elimination of any active cholinesterase therefrom. That is to say, layer 4 can be produced from a sheet manufactured as described above but without binding the cholinesterase to the sulfited, hydrophilic polyacrolein. Alternatively, the same sheet used to manufacture element 3 may be used, however, the active cholinesterase thereof can first be deactivated such as by heating the sheet to a temperature at which the enzyme is destroyed, by contacting the sheet with an inactivating material e.g. an anti-cholinesterase material and the like. The element can then be obtained from the sheet free of active cholinesterase but containing sulfited polyacrolein in any suitable manner e.g. punching, cutting etc. The outer diameter of layer 4 should be at least as large as the diameter of element 3 and the inner diameter thereof should be smaller than the diameter of

said element. If desired, it can be saucer shaped in the same manner as layer 6.

When layer 6 is employed it can be prepared from any material which, because of its stiffness, causes element 3 to be held in place over hole 2 in holder 1. Top ring 6 can therefore be made of paper, cardboard, plastic, glass, metal etc. and is preferably shaped like an inverted dish so as to hold layer 4 and element 3 in place and be capable of being attached to substrate 1, such as with an adhesive. When layer 6 is used in conjunction with layer 4, it is preferred that layer 6 have both an inner diameter and an outer diameter larger than those of layer 4. The inner diameter of layer 6, however, not exceeding the outer diameter of layer 4.

Assembled, element 3 is positioned over hole 2 of holding means 1. Layer 4 is placed atop element 3 with its inner hole preferably aligned with that of the substrate. Clamping layer 6 is then positioned over layer 4 and the whole unit is tightly combined such as by placing clamps, not shown, on means 1 over the lip edge 8 of layer 6.

In use, cellulosic element 3, having the sulfited polyacrolein bound active cholinesterase dispersed throughout the interstices thereof, and layer 4, free of active cholinesterase, are wetted with a "buffer" solution having a pH in the range 7.0–7.5. Suitable materials for this purpose include water, tris(hydroxymethyl)aminomethane, phosphate buffer and the like.

A preferred material is a solution containing 2.78% of monobasic sodium phosphate and 5.4% of dibasic sodium phosphate.

After element 3 and layer 4 are wetted with the buffer solution, the device is exposed to the environment to be sampled and are then contacted with N-methyl indoxyl butyrate. The butyrate, normally being a solid, is preferably used in the form of a solution, the solvent of which is preferably a secondary or tertiary alcohol, such as isopropyl alcohol, isoamyl alcohol, 2-butanol, t-butyl alcohol, mixtures thereof and the like. The use of other solvents is tolerable, however, but the N-methyl indoxyl buytrate exhibits long term stability in the above alcohols whereas its stability in other solvents is less satisfactory.

When element 3 containing the sulfited polyacrolein bound active cholinesterase and active cholinesterase free layer 4 are wetted, their color ranges from a dark yellow to orange. The presence of any anticholinesterase materials in the vicinity of the wet components will, however, then deactivate the bound active cholinesterase. When N-methyl indoxyl butyrate is then added, element 4 and layer 4 will remain yellow to orange and the person conducting the test will know anticholinesterase materials are present.

However, if no anticholinesterase materials are present, the added N-methyl indoxyl butyrate will be hydrolyzed by the active cholinesterase in element 3. The hydrolyzed butyrate is colorless, however, over a period of from about 1–2 minutes, the hydrolyzed butyrate is oxidized by the oxygen in the atmosphere and the color of element 3 contrasts with the yellow of layer 4 and becomes a deep green, thereby indicating to the observer that the immediate area is free of anticholinesterase materials such as nerve gas, sarin, prostigmine, eserine, and the like. Layer 4, remains yellow to orange because it contains no active cholinesterase. The green color of element 3 is therefore clearly distinguishable from the yellow to orange color of layer 4 thereby unequivocally indicating the absence of anticholinesterase materials.

EXAMPLE A

Solubilization of polyacrolein

To a suitable reaction vessel fitted with stirrer, condenser, thermometer, nitrogen gas inlet and constant temperature bath are added 344 parts of sodium metabisulfite and 2400 ml. of distilled water. The pH of this solution is adjusted to 5.6 with 10 M sodium hydroxide solution and 300 parts of finely divided polyacrolein are added. The reaction is allowed to continue under a nitrogen blanket at 65° C. until a clear, viscous, water-soluble polyacrolein adduct forms. The reaction mixture is cooled and stored.

EXAMPLE B

Cross-linking of soluble polyacrolein

To a suitable glass-lined reaction vessel equipped with stirrer and nitrogen gas inlet are added 2500 ml. of the polyacrolein bisulfite adduct produced in Example A, above, in 4000 ml. of distilled water. The solution is gently stirred and 300 parts of hexamethylene diamine in 400 ml. of distilled water are added drop-wise over a 4 hour period. A yellow cross-linked polymer becomes suspended and is heated to 60° C. under a heavy nitrogen blanket for 10 minutes and then cooled to room temperature. The polymer is filtered through cheesecloth, placed in a Büchner funnel and washed thoroughly with water. The cross-linked polymer is then slurried gently with 10 times its volume of water for 15–20 minutes, allowed to stand 20 minutes and is filtered. The washing procedure is repeated until the pH of the washings are between 6.5 and 7.0. The solid adduct is then slurried gently for 20 minutes with 1.0 M disodium phosphate adjusted to pH 6.5 and washed with distilled water.

The following examples are set forth for purposes of illustration only and are not to be construed as limitations on the instant invention except as set forth in the appended claims. All parts and percentages are by weight unless otherwise specified.

EXAMPLE 1

To a suitable reaction vessel is added 0.625 part of horse serum cholinesterase and 50 ml. of phosphate buffer (0.02 M; pH 7.4). The solution is allowed to stand in the refrigerator without agitation for 30 minutes. The enzyme is then completely dissolved by stirring. In a separate vessel are slurried 25 parts of the modified polyacryolein of Example B above (14 mg./g. binding capacity) with 50 ml. of the same phosphate buffer. After stirring 10 minutes, the pH is readjusted to 7.4 with 0.1 N sodium hydroxide. The contents of both vessels are then admixed and allowed to stir gently overnight at 40° C. The enzyme adduct is then filtered and washed with copious amounts of deionized water. Consistent binding yields of 75–95% are achieved using this method.

A 50/50 Albacel/Astracel pulp (concentration 2.6 g./100 cc.) is washed with water and subsequently with methanol to remove any residual sulfite and dried. To 1.3 parts of pulp are added 5.0 parts of wet enzyme adduct (100 units/g.) in a suitable blending vessel. The ingredients are blended for 5 minutes, ice being added to the mixture to prevent heat build-up. The blended slurry is then processed into a paper mat about the thickness of standard filter paper and of six inch diameter on a British Hand Sheet Mold. The paper is then dried in vacuo over a desiccant for 16 hours. The resultant dry sheet yields 236 ½ inch discs with approximately 0.6 unit of activity each.

One of the ½ inch discs is then placed over the ⅜ inch perforation in a sheet of .050 mil. cardboard. A ring of ½ inch outside diameter and ⅜ inch inside diameter is then placed over the disc. The ring is produced in exactly the same manner as the disc except that the modified polyacrolein of Example B is not treated with horse serum cholinesterase before being incorporated into the pulp.

A second ring of clear acrylic plastic shaped like an inverted dish is then placed over the assembly and cemented to the substrate with acrylic cement. The outer diameter of the plastic ring is ¾ inch while the inner diameter is 7/16 inch.

The ½ inch disc and ½ inch ring are then wetted with a phosphate buffer of pH 7.4. Both turn orange. Both wetted items are then contacted with a solution of

N-methyl indoxyl butyrate in isopropanol. The color change is observed as set forth in Table I, below.

TABLE I

Color		Total elapsed time
Disc	Ring	
Orange	Orange	0 min. 8.4 sec.
Light green	do	1 min. 0.5 sec.
Green	do	2 min. 0.4 sec.
Dark green	do	4 min. 3.8 sec.

EXAMPLE 2

When the acrylic plastic ring of Example 1 is replaced by a similarly shaped ring of identical size and produced from the same materials as the disc but free of active cholinesterase (the smaller ring having been omitted) similar results are observed.

EXAMPLE 3

When the above procedure of Example 1 is again followed and the buffer and N-methyl indoxyl butyrate solutions are added to the disc and ring in the presence of a gaseous anticholinesterase material, both the disc and ring remain orange after 15 minutes of exposure to the atmosphere.

EXAMPLES 4–8

Sulfiting the polyacrolein of Example A with (4) sodium sulfite, (5) sodium hydrosulfite, (6) sulfurous acid, (7) calcium sulfite or (8) ammonium bisulfite and using the resultant material as in Examples B and 1 does not alter the orange to green color change shown above in Table I.

EXAMPLES 9–11

Cross-linking the polyacrolein with (9) isophorone diamine, (10) 4-aminophenyl sulfide hydrochloride salt and (11) methylenebisacrylamide as in Example B and utilization of the resultant material as in Example 1 does not change the orange to green color variation shown in Table I, above.

EXAMPLES 12 AND 13

Replacement of the pulp of Examples 1 and 3 with a bleached kraft pulp does not alter the detection ability of the devices used therein.

EXAMPLE 14

The procedure of Examples 1 and 3 are again followed except that the N-methyl indoxyl butyrate is used as a solution in isoamyl alcohol. Similar results are achieved.

I claim:

1. An article of manufacture comprising, in superimposed relationship, (1) a self-supporting layer, (2) a cellulosic element having active cholinesterase bound to sulfited polyacrolein dispersed throughout the interstices thereof and (3) an active cholinesterase free cellulosic layer having at least one perforation therein and having the same physical properties of color and texture as cellulosic element (2) after both have been wetted.

2. An article according to claim 1 including (4) a second layer having at least one perforation therein aligned with, larger than and positioned atop that of said (3).

3. An article according to claim 1 including means for maintaining the integrity of said article.

4. An article according to claim 2 including means for maintaining the integrity of said article.

5. An article according to claim 1 wherein said layer (3) is dish-shaped.

6. An article according to claim 2 wherein said layer (4) is dish-shaped.

7. An article according to claim 1 wherein said layer (3) has sulfited polyacrolein dispersed throughout the interstices thereof.

8. An article according to claim 2 wherein said layer (3) has sulfited polyacrolein dispersed throughout the interstices thereof.

9. An article according to claim 1 wherein said (1) is cardboard.

10. An article according to claim 2 wherein said (1) is cardboard.

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DAVID M. NAFF, Examiner

U.S. Cl. X.R.

23—753 TP; 195—103.5 R, 63, DIG 11